Biochimica et Biophysica Acta 1843 (2014) 508–516

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Ionizing radiation-inducible miR-494 promotes glioma cell invasion through EGFR stabilization by targeting p190B RhoGAP

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ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 22 November 2013
Accepted 26 November 2013
Available online 4 December 2013

Keywords:
MicroRNA
Invasion
miR-494
EGFR
p190B RhoGAP

ABSTRACT

MicroRNAs (miRNAs) play an important role in various stages of tumor progression. miR-494, which we had previously identified as a miRNA induced by ionizing radiation (IR) in the glioma cell line U-251, was observed to enhance invasion of U-251 cells by activating MMP-2. The miR-494-induced invasive potential was accompanied by, and dependent on, epidermal growth factor receptor (EGFR) upregulation and the activation of its downstream signaling constituents, Akt and ERK. The upregulation of EGFR by miR-494 involved the suppression of lysosomal protein turnover. Among the putative target proteins tested, p190B RhoGAP (p190B) was downregulated by miR-494, and its reduced expression was responsible for the increase in EGFR expression. A reporter assay using a luciferase construct containing p190B 3′-untranslated region (3′UTR) confirmed that p190B is a direct target of miR-494. Downregulation of p190B by small interfering RNA (siRNA) transfection closely mimicked the outcomes of miR-494 transfection, and showed increased EGFR expression, MMP-2 secretion, and invasion. Ectopic expression of p190B suppressed the miR-494-induced EGFR upregulation and invasion promotion, thereby suggesting that p190B depletion is critical for the invasion-promoting action of miR-494. Collectively, our results suggest a novel function for miR-494 and its potential application as a target to control invasiveness in cancer therapy.

1. Introduction

The spread of cancer cells into neighboring tissues is termed invasion, and is one of the hallmarks of cancer malignancy. High invasiveness is closely related to the aggressiveness and poor prognosis of tumors. Invasion and metastasis are also closely associated in that cancer cells first invade connective tissue and penetrate into blood vessels to initiate metastasis. Malignant gliomas are typically extremely aggressive tumors that have high invasiveness. Local invasive growth of gliomas complicates complete surgical resection, thereby yielding a high incidence of recurrence with a poor survival rate [1]. In addition to migratory activity, proteolytic degradation of the extracellular matrix (ECM) is a critical element of cancer cell invasion, since the ECM is a barrier to invading cancer cells. Cancer cells produce a number of extracellular proteases, such as matrix metalloproteinases (MMPs) and the urokinase-type plasminogen activator (uPA), to degrade ECM components and enable penetration into surrounding tissues. Cancer invasion is controlled by the local microenvironment and involves autocrine and paracrine factors such as epidermal growth factor (EGF) and transforming growth factor β (TGFβ), respectively [2,3]. Among the various signaling components, the members of the receptor tyrosine kinase (RTK) family, which include epidermal growth factor receptor (EGFR), play a central role in the regulation of invasion [2,4,5].

The activation of EGFR, which is a member of the HER-erbB family of RTKs, triggers the activation of various intracellular signaling pathways, including Ras/mitogen activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, that regulate a variety of processes such as proliferation, survival, invasion, and metastasis of cancer cells [6]. EGFR is commonly overexpressed in gliomas and has been shown to mediate ionizing radiation (IR)-induced MMP-2 secretion and invasion via Akt activation [7]. EGFR signaling is often hyperactivated in multiple cancer types by several mechanisms such as activating mutations in the kinase domain, gene amplification, and overexpression of EGFR and its ligands [8–11]. Once activated by the ligands, EGFR signaling is also regulated by a negative feedback mechanism, which involves endocytosis and subsequent degradation of EGFR in the lysosome, thereby leading to the termination of receptor signaling [12]. This process is regulated at various stages by many proteins including a small GTPase, Rab5A, and a guanine nucleotide exchange factor (GEF) for the Rho family GTPases, Vav2 [13,14]. Importantly, deregulation of this process can lead to abnormal EGFR turnover and signaling.

Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor (EGFR); FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; IR, ionizing radiation; LNA, locked nucleic acid; MAPK, mitogen activated protein kinase; miRNA, microRNA; MMP, metalloproteinase; NLK, Nemo-like kinase; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; qRT-PCR, quantitative real-time polymerase chain reaction; RTK, receptor tyrosine kinase; SDS, sodium dodecyl sulfate; ST7L, suppression of tumorigenicity 7-like; TGFβ, transforming growth factor β; 3′UTR, 3′-untranslated region; uPA, urokinase-type plasminogen activator

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http://dx.doi.org/10.1016/j.bbamcr.2013.11.021

MicroRNAs (miRNAs), which are a class of endogenous small non-coding RNAs of ~22 nucleotides in length, negatively regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated region (3′UTR) of target mRNAs [15]. In addition, miRNAs play an important role in a variety of biological processes such as proliferation, apoptosis, and tumorigenesis [16,17]. The abnormal expression of miRNAs is a characteristic feature of cancers, and is involved in various stages of tumorigenesis [18]. An increasing number of studies have examined the roles of miRNAs in the response of cancer cells to IR, which is an important genotoxic modality that is widely used for the treatment of cancers. Notably, a number of miRNAs have been identified that are regulated by IR and can modulate sensitivity to radiotherapy [19].

We have recently identified miR-494 as one of the IR-inducible miRNAs in the glioma cell line U-251 by using microarray analysis of the miRNA expression profile [20]. miR-494 did not significantly affect the growth or radiosensitivity of U-251. Here, we report that miR-494 enhances invasion by upregulating EGFR expression and the subsequent activation of downstream ERK and Akt signaling. Further investigation revealed that p190B RhoGAP (p190B) was the direct target of miR-494, the downregulation of which was responsible for EGFR upregulation by miR-494 through protein stabilization. This is a newly identified mechanism for regulating EGFR expression during the induction of invasion that could be exploited to develop a novel anti-invasion strategy.

2. Materials and methods

2.1. Cell culture and reagents

The human glioma cell line, U-251, was grown in ME medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (10 μg/mL) at 37 °C in a humidified incubator containing 5% CO_2. Antibodies against phospho-EGFR (9H2), EGFR (1005), EGFR (528), p190B (54), SOCS-6 (H-251), Rab5A (S-19), uPA, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Akt, Akt, phospho-ERK, ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA), and the MMP-2 antibody was from Calbiochem (La Jolla, CA). AG1478, LY294002, and PD98059 were also purchased from Calbiochem.

2.2. Plasmid constructs

The expression vector for p190B was constructed by in-frame cloning into the p3XFlag-Myc-CMV vector after PCR amplification of the human *p190B* open reading frame (ORF) using the primers 5′-CGGC GGCCGCGATGATGGCAAAAAAC-3′ (forward) and 5′-CCGAATTCTCAT ATAATGCCAAGAGG-3′ (reverse). The expression vector for the dominant negative (DN, T19N) RhoA was kindly provided by Dr. Park J.B. (Kangwon National University, Korea). To prepare the reporter constructs, DNA fragments of *p190B* 3′UTRs containing the putative miR-494 binding sites were cloned into pGL3luc, which was kindly provided by Dr. Kim (Seoul National University, Korea). The primers for cloning the *p190B* 3′UTRs were as follows: *p190B* 3′UTR-1, 5′-AGTCTAGAGA AGTGATTGCAAACAGG-3′ (forward) and 5′-AGGAATTCAGTTATCTAC ACCGACTGC-3′ (reverse); *p190B* 3′UTR-2, 5′-AGTCTAGATGGTAAGTTA CTGGAAGG-3′ (forward) and 5′-AGGAATTCAGATTGCGGTTACCTGC-3′ (reverse). Mutant reporter vectors were generated by introducing 3 nucleotide mutations into the putative miR-494 binding sites using a QuickChange® II Site-Directed mutagenesis kit (Agilent Technologies).

2.3. Oligoribonucleotides and transfection

Synthetic miRNA mimics were synthesized by Samchully Pharmaceutical (Seoul, Korea) as RNA duplexes designed from the sequences of 5′-UGAAACAUACACGGGAAACCUC-3′ (miR-494) and 5′-CUUACGCU GAGUACUUCGATT-3′ as a negative control of miRNA. An inhibitor of miR-494 was a 2′-*O*-methyl-modified oligoribonucleotide single strand with a sequence of 5′-GAGGUUUCCCGUGUAUGUUUCATT-3′. Stealth siRNA duplex oligonucleotides targeting p190B, Rab5A, and EGFR, and scrambled non-targeting negative control siRNA were purchased from Santa Cruz Biotechnology. For functional analyses, the cells were transfected with miRNA or siRNA oligonucletides at a final concentration of 20 nM by using G-fectin (Genolution, Seoul, Korea) or Lipofectamine® RNAiMAX (Invitrogen) according to the manufacturer's recommendation.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using a Trizol reagent (Molecular Research Center Inc.). Two micrograms of RNA was transcribed into single-stranded cDNA using Superscript II (Invitrogen), and used for PCR amplification. qRT-PCR was performed using the SYBR premix Ex Taq II (TaKaRa) and the iCycler real-time PCR detection system (Bio-Rad). The sequences of the primers were as follows: EGFR, 5′-GGACTCTGGA TCCCAGAAGGTG-3′ (forward) and 5′-GCTGGCCATCACGTAGGCTT-3′ (reverse); MMP-2, 5′-ACAAAGAGTGGCAGTGCAA-3′ (forward) and 5′-CACGAGCAAAGGCATCATCC-3′ (reverse); p190B, 5′-AGCCAAATTCCT GCCAATAAG-3′ (forward) and 5′-AGGAAGGGTGAAAGAATAAGATCCA-3′ (reverse). qRT-PCR for miRNA quantification was performed using the TaqMan MicroRNA assay kit (Applied Biosystems) and miR-494-specific primers according to the manufacturer's instruction. We used β-actin and U6 spliceosomal RNA as internal normalizers for mRNA and miRNA, respectively.

2.5. Reporter assay

The cells were transfected with reporter plasmid (100 ng), pRL-CMV-*Renilla* plasmid (2 ng), and miRNA using Lipofectamine® 2000 (Invitrogen). After 48 h of transfection, luciferase assays were conducted using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instruction. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

2.6. Western blotting

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. After the transfer, the membranes were blocked in 5% skim milk in Tris–buffered saline containing Tween 20 (TBST; 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h, and incubated with the specific primary antibody in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed 3 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence detection using an enhanced chemiluminescence system (ECL; Amersham).

2.7. Wound healing and invasion assay

At 48 h after transfection, the confluent cell monolayer was scratched across the well using a 200 μL tip and the medium was exchanged with fresh medium. After incubation for 16 h to allow migration, the cells were washed twice with PBS, and fixed with 3.7% paraformaldehyde for 30 min followed by staining with 1% crystal violet in 10% ethanol for 30 min. Invasion assays were performed using Transwell® Permeable Supports (Corning). Cells (2×10^4) in 200 μL of medium were placed onto the upper surface of Matrigel-coated filters in Boyden chambers. The lower chambers were filled with 1 mL of serum-free medium supplemented with 0.1% BSA. After 20 h of incubation, the cells that had migrated to the lower surface of the filter were fixed and stained using a Diff-Quick stain kit (Polyscience). The stained cells were counted using a microscope.

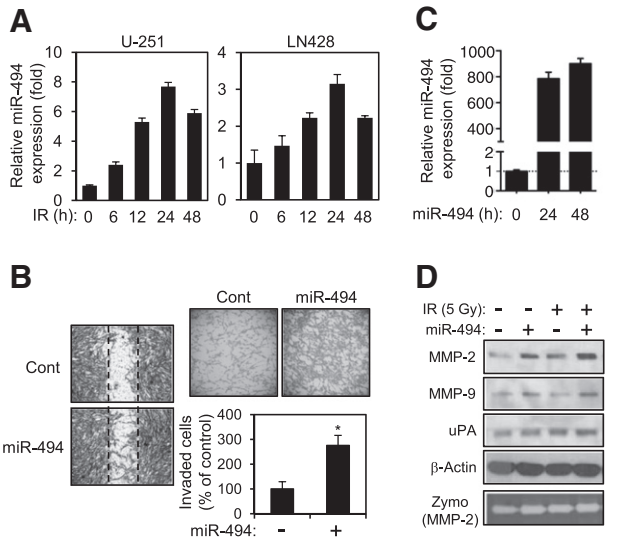

Fig. 1. IR-inducible miR-494 enhances cell migration, invasion, and MMP2 secretion. (A) miR-494 expression is induced by IR. Total RNA was extracted from U-251 and LN428 cells at the indicated times after exposure to IR, and miR-494 expression levels were quantified by qRT-PCR. The means of triplicate experiments were plotted and analyzed for statistical significance by the Student's *t*-test. (B) miR-494 enhances migration and invasion of U-251 cells. The monolayers of the cells transfected with miRNA were scratched and the cells were stained 16 h after scratching (left panel). Transfected cells were seeded onto Matrigel-coated inserts in modified Boyden chambers and processed as described in the Section 2. *, $P < 0.001$ ($N = 3$). (C) Determination of miR-494 level after transient transfection with miR-494. U-251 cells were transfected with synthesized miR-494 mimic at 20 nM, and total RNA was extracted at the indicated times after transfection to quantify miR-494 level. (D) miR-494 enhances MMP secretion. The abundance of MMP-2, MMP-9, and uPA in the conditioned medium was determined by Western blot analysis, and the MMP-2 activity was assessed by gelatin zymography.

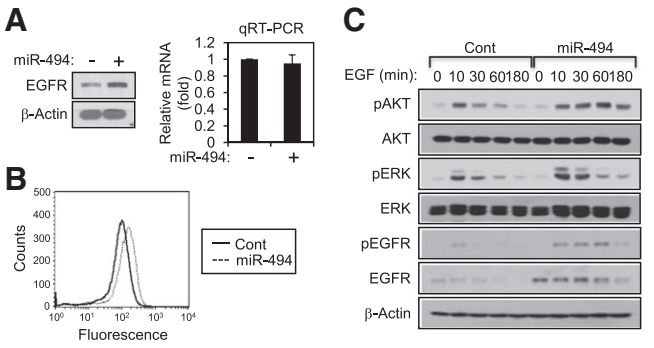

Fig. 2. miR-494 upregulates EGFR expression and activates EGFR signaling. (A) miR-494 induces upregulation of EGFR protein levels. The cell lysates and total RNA were prepared from the U-251 cells 48 h after transfection with control or miR-494. The protein and mRNA levels of EGFR were determined by Western blot and qRT-PCR analysis. miR-494 upregulates EGFR protein levels, but not EGFR mRNA levels. (B) miR-494 upregulates the abundance of EGFR on the cell surface. The surface EGFR was stained using a FITC-labeled antibody specific for the exposed part of EGFR, and the cells were analyzed by FACS. (C) miR-494 activates EGFR downstream signaling. Transfected cells were serum-starved for 16 h, then stimulated with EGF (100 ng/mL). The cell lysates were prepared at the indicated times after EGF treatment and subjected to Western blot analysis.

2.8. Gelatin zymography

The gelatin-digesting activity of the media was determined to assess the activity of the MMPs secreted into the media. Cells were incubated in serum-free media for 24 h to prepare the conditioned media. The conditioned media were mixed with 2× non-reducing sample buffer and subjected to 10% SDS-PAGE containing 0.1% gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h, incubated in gelatinase developing buffer for 16 h at 37 °C, and stained with 0.1% Coomassie brilliant blue. The MMPs activities were visualized as clear bands against the stained background.

2.9. Determination of EGFR on cell surface

At the indicated times after stimulation with EGF (100 ng/mL), the cells were harvested in ice cold PBS containing 10% FBS and 1% sodium azide. The FITC-labeled antibody specific for surface EGFR (528; Santa Cruz) was added in the cell suspension, and incubated for 30 min at 4 °C. The cells were washed with cold PBS, resuspended in ice cold PBS containing 10% FBS and 1% sodium azide, and then analyzed using a FACS Calibur apparatus (BD Biosciences).

2.10. Determination of RhoA activation

RhoA activation was assessed by RhoA pull-down assays by using a RhoA activation assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instruction. In brief, Rhoetekin RBD agarose beads were added to the cell lysates, and incubated at 4 °C for 1 h with gentle agitation. After thorough washing, the proteins bound to the beads were eluted in 2× reducing SDS-PAGE sample buffer, and subjected to Western blot analysis.

2.11. Irradiation of cells

Cells were exposed to γ-rays by using a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Canada) with a dose rate of 3.81 Gy/min.

3. Results

3.1. miR-494 is induced by IR, and enhances cell migration and invasion

In an effort to identify IR-responsive miRNAs using miRNA microarray analysis, we previously identified miR-494 as one of the IR-inducible miRNAs in the glioma cell line U-251 [20]. Here, we measured miR-494 expression at several time points after IR treatment in 2 glioma cell lines by miRNA qRT-PCR to validate the previous microarray data. Indeed, we confirmed that IR induced an 8-fold increase in miR-494 expression at 24 h after IR treatment in U-251 cells, while LN428 exhibited a lower increase of miR-494 expression after IR treatment (Fig. 1A). In the previous study, analysis of the various IR-linked cellular responses demonstrated that miR-494 does not significantly affect cell growth or apoptosis. Based on the report that IR enhances invasion of glioma cells [7], we decided to examine whether IR-inducible miR-494 plays a role in invasive potential. Firstly, we performed wound-healing assays to assess the effect of miR-494 expression on cellular migratory activity. Transfection with miR-494 enhanced the migration of U-251 cells. The invasion assay using Matrigel-coated transwells showed that miR-494 transfection resulted in markedly increased invasive activity of U-251 cells (Fig. 1B). We checked miR-494 level after transient transfection with 20 nM miRNA mimic, which is a standard transfection condition we used for migration and invasion experiments. The qRT-PCR result showed that miR-494 level was increased by several hundred-fold compared to the control (Fig. 1C). We next examined the effect of miR-494 on the expression of extracellular proteases that are known to play an important role in invasion. Transfection with miR-494 resulted in a marked increase in MMP-2 secretion with a weak effect on the activation of MMP-9 and uPA (Fig. 1D). IR alone also caused an increase in MMP-2 activation as expected from the previous report [7], and the combined treatment of IR and miR-494 transfection induced an even greater increase in MMP-2 secretion. The zymograph showing the enzyme activity of secreted MMP-2 also confirmed the effect of miR-494 on MMP-2. Taken together, miR-494 efficiently activates cellular invasion and the secretion of extracellular proteases, in particular MMP-2.

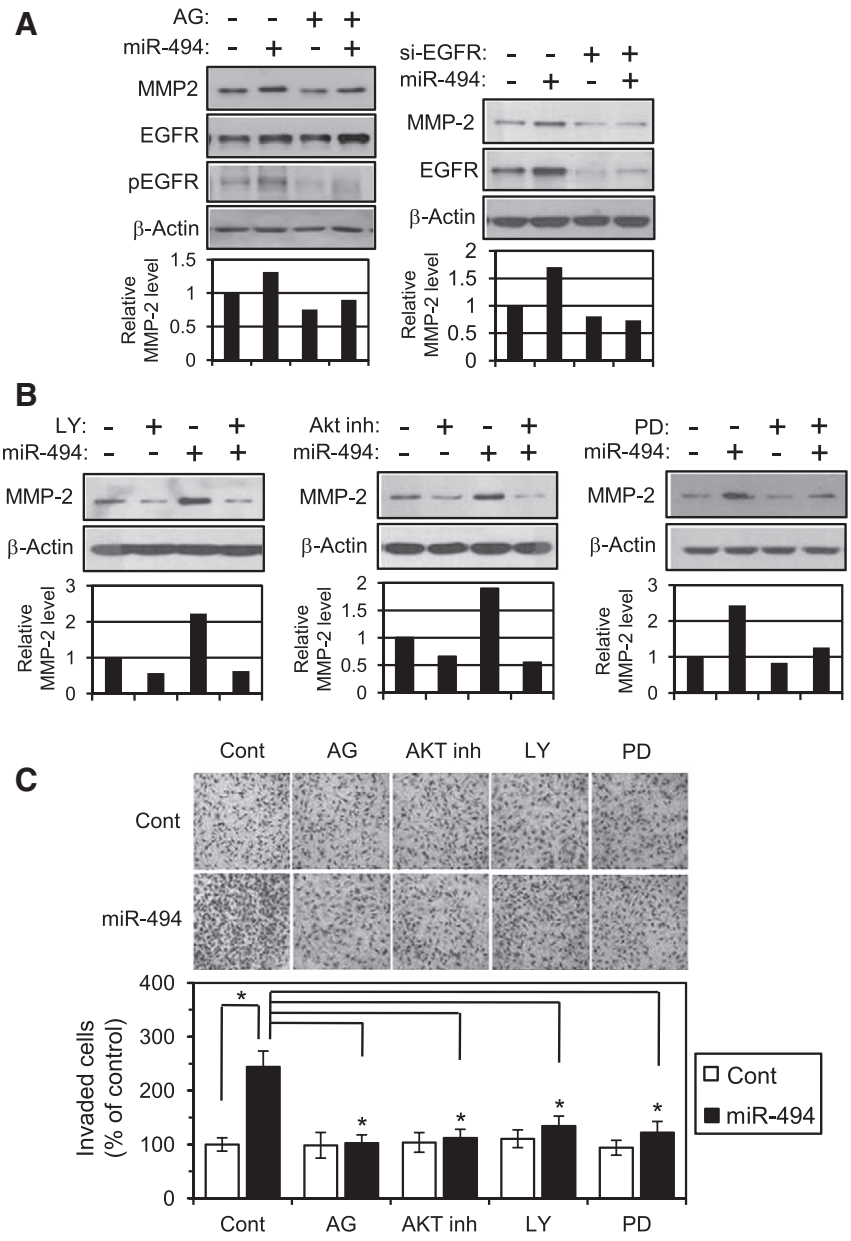

Fig. 3. Enhancement of MMP-2 secretion and invasion by miR-494 is dependent on EGFR, PI3K/Akt, and ERK activation. (A) Enhancement of MMP-2 secretion by miR-494 is dependent on EGFR. Conditioned media were prepared from the transfected cells after treatment with the EGFR kinase inhibitor AG 1478 (AG, 10 μM) or the cells cotransfected with siRNA specific for EGFR (si-EGFR). Western blot analyses were performed to determine the MMP-2 levels in the conditioned media. Densitometric intensities of the bands normalized to that of β-actin are plotted for better comparison. (B) Enhancement of MMP-2 secretion by miR-494 is dependent on the downstream signals of the PI3K/Akt and ERK pathways. Transfected cells were treated with LY294002 (LY, 10 μM), Akt inhibitor I (Akt inh, 10 μM), and PD98059 (PD, 10 μM), and the conditioned media were prepared and analyzed for the MMP-2 levels. (C) Promotion of invasion by miR-494 is dependent on EGFR, PI3K/Akt, and ERK activation. Cells were pretreated with the inhibitors for 2 h before reseeding, and maintained with the inhibitors during the invasion assays. *, $P < 0.001$ ($N = 3$).

3.2. miR-494 upregulates EGFR expression and enhances the activation of downstream effectors of EGFR signaling

It has been previously reported that IR enhances MMP-2 secretion and invasion through the EGFR signaling pathway [7]. Therefore, we examined whether EGFR signaling is involved in the invasion-promoting action of IR-inducible miR-494. Firstly, we determined the effect of miR-494 on the expression of EGFR. Transfection with miR-494 increased the protein levels of EGFR, while the mRNA expression of EGFR was not significantly altered by miR-494 (Fig. 2A). This observation strongly suggests that miR-494 induces the upregulation of EGFR expression by a posttranscriptional mechanism, such as protein stabilization. Fluorescence-activated cell sorting (FACS) analysis after staining the cell surface EGFR showed that the upregulation of EGFR expression induces increased numbers of the receptor proteins that are exposed on the cell surface and ready to be activated (Fig. 2B). Next, we examined the effects of miR-494 on the activation of EGFR and its typical mediators of downstream signaling. Compared to the control, phosphorylation of EGFR in the miR-494-transfected cells was markedly enhanced and lasted long after stimulation by EGF treatment (Fig. 2C). As expected from this finding, ERK activation was also significantly augmented by transfection with miR-494. The basal Akt phosphorylation level in the miR-494-transfected cells was higher and maintained longer after stimulation than in the control. Collectively, these data suggest that EGFR signaling is a plausible candidate mediator for miR-494 to enhance invasion through MMP-2 activation.

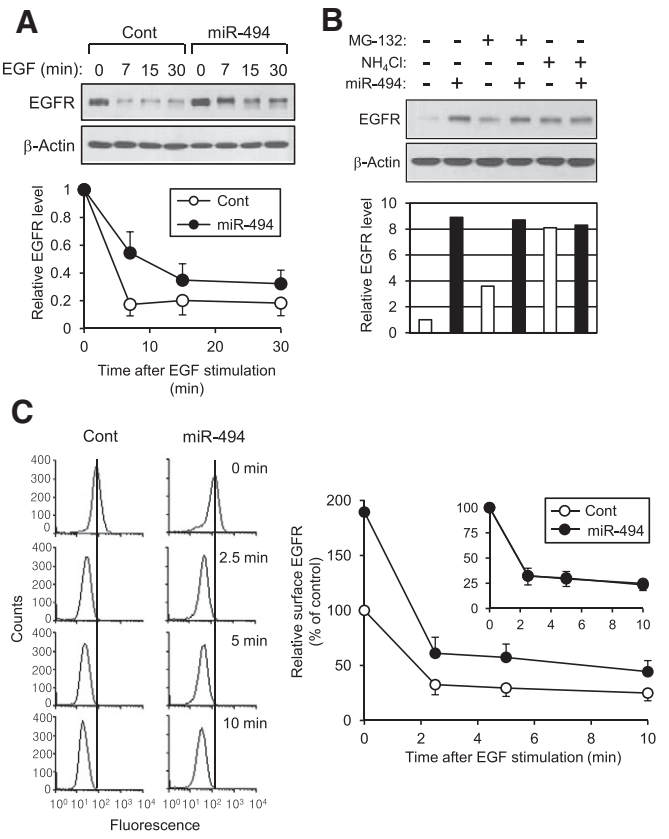

Fig. 4. miR-494 upregulates EGFR expression by protein stabilization without affecting kinetics of EGFR endocytosis. (A) miR-494 attenuates the turnover of EGFR. The cells transfected with control or miR-494 were pretreated with cycloheximide (CHX, 20 μg/mL) for 1 h to block protein synthesis, and then stimulated with EGF (100 ng/mL). The cells were harvested at the indicated times and analyzed by Western blot. The normalized band intensities were plotted against time after stimulation to determine the kinetics of EGFR degradation. The data were obtained from 3 independent experiments. (B) EGFR upregulation by miR-494 is mainly through the suppression of lysosomal degradation. The cells transfected with control or miR-494 were treated with MG-132 (20 μM) or NH_4Cl (200 mM) for 12 h before cell harvesting. Western blot analysis was performed to determine the EGFR levels. Densitometric intensities of the bands normalized to that of β-actin are shown. (C) The kinetics of EGFR endocytosis is not affected by miR-494. The cells were stimulated with EGF (100 ng/mL), and stained for surface EGFR at the indicated intervals. The fluorescence intensities of the peaks were plotted against time after stimulation. The insert in the upper right shows the plots after the starting level for each is set to 100%.

3.3. Enhancement of invasion and MMP-2 secretion by miR-494 is dependent on the activation of EGFR, PI3K/Akt, and ERK

To investigate the role of EGFR signaling in the invasion-promoting action of miR-494, we first tested whether inhibition of EGFR signaling suppresses the miR-494-induced increase in MMP-2 activation. The treatment with AG 1478, which is a specific inhibitor of EGFR, completely blocked the miR-494-induced increase in MMP-2 secretion. Knocking-down EGFR expression by transfection with EGFR-specific siRNA also exhibited a similar effect (Fig. 3A). Inhibition of Akt activation by treatment with LY294002 or an Akt inhibitor caused the decrease in basal MMP-2 secretion and also blocked the miR-494-induced increase in MMP-2 secretion (Fig. 3B). Blocking ERK activation by treatment with the MEK1 inhibitor PD98059 also resulted in significant suppression of the MMP-2 secretion enhanced by miR-494. The effects of the inhibition of EGFR signaling on the action of miR-494 were also assessed by invasion assay after treatment with these inhibitors. Similarly to the effect on MMP-2 secretion, inhibiting the activation of EGFR, Akt, and Erk significantly suppressed miR-494-induced invasion (Fig. 3C).

3.4. miR-494 upregulates EGFR expression by protein stabilization

Our previous observation that miR-494 caused the increase in EGFR protein levels without altering the mRNA expression (Fig. 2A) suggests that miR-494 upregulates EGFR levels by regulating the protein stability. Following activation as a result of binding to the ligand EGF, EGFR undergoes endocytosis and subsequent degradation to terminate receptor signaling. To examine whether miR-494-induced EGFR upregulation is associated with alterations to the degradation process, we compared the kinetics of EGFR degradation after stimulation with EGF. In the control cells, the EGFR levels rapidly decreased to the lowest values (about 20% of the untreated) within 7 min after EGF stimulation. In contrast, EGFR degradation after EGF stimulation was significantly delayed in the cells transfected with miR-494; more than 50% EGFR remained at 7 min and a higher lowest level was maintained (Fig. 4A). This result indicates that miR-494 attenuates the turnover of EGFR. To examine which proteolytic processes are affected by miR-494, we determined the EGFR level after miR-494 transfection and treatment with MG-132 and NH_4Cl, which are inhibitors of proteasomal and lysosomal protein degradation, respectively. Treatment with MG-132 caused slightly increased basal EGFR levels, but the EGFR expression in the miR-494-transfected cells was still significantly higher than the control (Fig. 4B). In contrast, NH_4Cl treatment resulted in the enhancement of the EGFR level of the control to that of the miR-494-transfected cells. This result strongly suggests that miR-494 induces EGFR upregulation by interfering mainly with the lysosomal protein degradation pathway. Next, we determined the kinetics of EGFR internalization after stimulation with EGF by staining EGFR exposed on the cell surface followed by FACS analysis. After the cells were stimulated with EGF, the distribution of the cells stained for surface EGFR was shifted toward lower stain intensity, which represents the internalization of EGFR (Fig. 4C). The intensity values of the peaks were plotted against time to compare the kinetics of EGFR internalization. In both the control and miR-494-transfected cells stimulated with EGF, the surface EGFR levels quickly decreased to about 30% of those untreated within 2.5 min, and very slowly decreased subsequently (Fig. 4C, right). After setting the starting surface EGFR level of each to 100% (insert), the plots clearly overlapped, thereby indicating that the kinetics of EGFR internalization were almost identical. These results suggest that miR-494 does not affect EGFR endocytosis, but does inhibit lysosomal degradation of EGFR after activation by EGF.

3.5. miR-494 upregulates EGFR expression by directly targeting p190B RhoGAP

To identify the target protein associated with the EGFR-stabilizing and invasion-promoting effects of miR-494, we shortlisted the putative target genes of miR-494 predicted by the miRNA target prediction programs TargetScan and DIANA-microT 3.0. Various proteins have been reported to be involved in the regulation of EGFR stability [13,14,21–23]. From the list of predicted target candidates, we selected 3 proteins that were expected to have a negative effect on EGFR stability to validate as a target of miR-494. We first determined the expression level of the candidate proteins after transfection with miR-494 in U-251 and HeLa cells. In both cell lines, the protein levels of p190B and Rab5A were reduced when the cells were transfected with miR-494, while the expression of SOCS-6 was not altered (Fig. 5A). To evaluate whether p190B or Rab5A is the target protein responsible for the upregulation of EGFR by miR-494, we next examined the effect of knocking-down the proteins by siRNA transfection on the expression level of EGFR. Knocking-down p190B expression resulted in increased EGFR levels, while knocking-down Rab5A expression did not alter the EGFR expression in either cell lines (Fig. 5B). Together with the downregulation of p190B expression by miR-494, the reproduction of EGFR upregulation by knocking-down p190B strongly suggests that p190B is the target for the EGFR-upregulating action of miR-494. The mRNA expression level of p190B

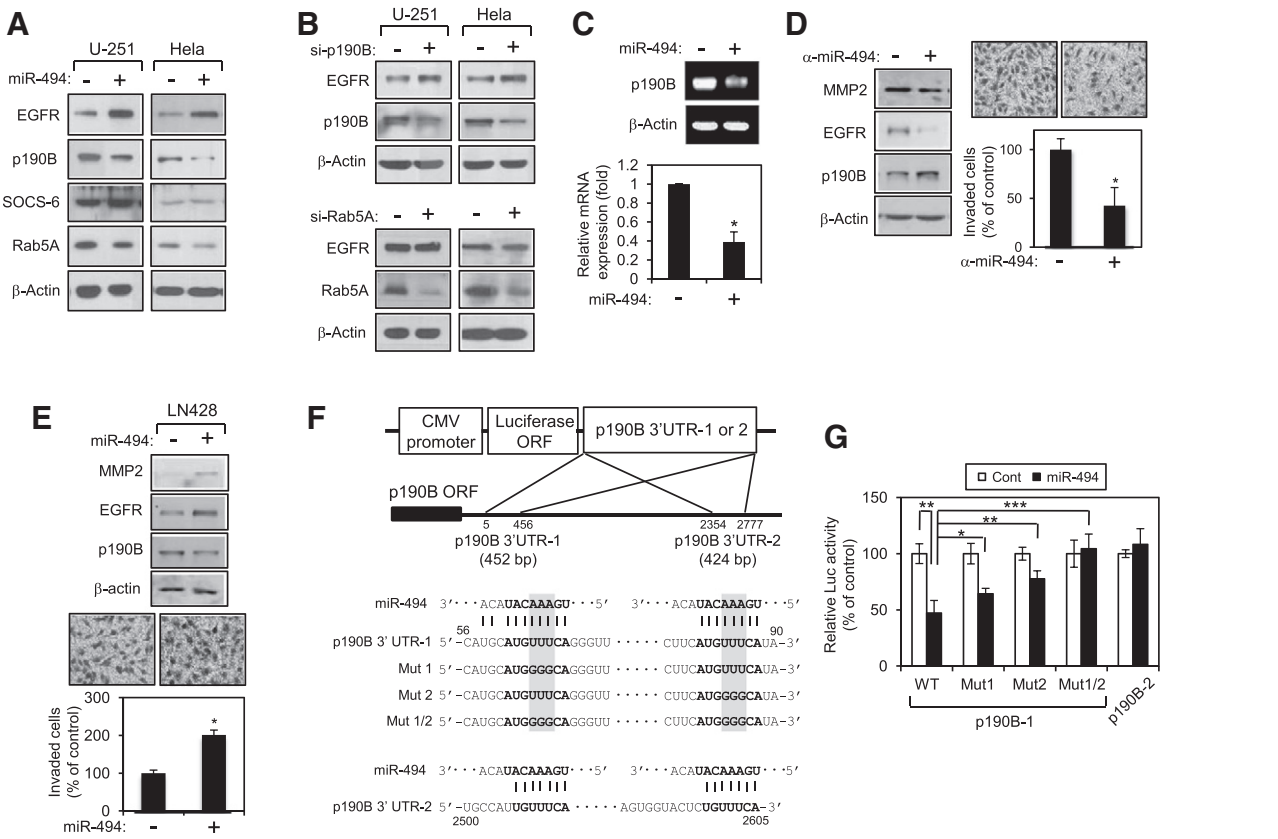

Fig. 5. miR-494 upregulates EGFR expression by targeting p190B. (A) miR-494 suppresses the expression of p190B and Rab5A, but not SOCS6. Cells were transfected with control or miR-494, and analyzed for the expression of the selected candidate target proteins. (B) Suppression of p190B expression induces the upregulation of EGFR expression. U-251 and Hela cells were transfected with the siRNAs specific for p190B (si-p190B) or Rab5A (si-Rab5A), then the cell lysates were prepared 48 h later, and analyzed for EGFR expression. (C) miR-494 reduces the expression of p190B mRNA. The mRNA levels were determined by conventional RT-PCR (upper) and qRT-PCR (lower) after transfection with miRNA. *, $P < 0.005$ ($N = 3$). (D) miR-494 inhibitor exhibits opposite effects of miR-494. U-251 cells were transfected with the miR-494 inhibitor and subjected to invasion assay or the cell lysates were prepared after 48 h. *, $P < 0.005$ ($N = 3$). (E) Confirmation of the effects of miR-494 in LN428. LN428 cells were transfected with miR-494 and incubated for 48 h. The procedure was the same to that for U-251. *, $P < 0.005$ ($N = 3$). (F) Structures of reporter constructs containing p190B 3′UTRs downstream of the luciferase ORF. The aligned sequences of p190B 3′UTRs complementary to the seed sequence of the miR-494 seed sequence and the mutant sequences are shown. The mutated regions are shaded. (G) Reporter assay showing the direct targeting of p190B 3′UTR by miR-494. U-251 cells were transfected with a combination of the indicated reporters and miRNAs, and the dual-luciferase assay was performed 48 h later. Firefly luciferase activity was normalized to *Renilla* luciferase activity. The data were obtained from 3 independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ ($n = 6$).

was also reduced by transfection with miR-494 (Fig. 5C). In contrast, inhibition of endogenous miR-494 using miR-494 inhibitor (α-miR-494) exhibited opposite effects of miR-494; downregulation of MMP2 secretion, reduction of EGFR expression and increase of p190B protein levels. The invasion ability of the cells was also decreased (Fig. 5D). We also confirmed the effect of miR-494 on p190B/EGFR/invasion axis using another glioma cell line, LN428. miR-494 led to the increase in MMP2 secretion, EGFR protein levels and invasion ability, as well as the suppression of p190B expression (Fig. 5E). Next, we tested whether p190B is a direct target of miR-494 by reporter assay. Four putative miRNA regulatory elements for miR-494 were found in the 3′UTR of p190B mRNA. To determine whether miR-494 directly targets these elements, we cloned 2 3′UTR fragments containing 2 miRNA regulatory elements each into the modified pGL3 reporter vector downstream of the firefly luciferase ORF (Fig. 5F). The 2 reporters were co-transfected into U-251 cells together with the control or miR-494, and the luciferase activity was measured 2 days after transfection. miR-494 strongly reduced the relative luciferase activity of the p190B-1 reporter to below 50% of the control, but not the p190B-2 reporter, thereby indicating that only p190B 3′UTR-1, which is located closer to the stop codon of p190B mRNA, is targeted by miR-494 (Fig. 5G). For further validation for the specificity and response of each miRNA regulatory element in targeting the p190B 3′UTR-1 by miR-494, mutant reporters containing a 3-nucleotide mutation introduced into each miRNA regulatory element were also prepared. The single mutations in each miRNA regulatory element partially suppressed the reduction of luciferase activity by miR-494, and the double mutation in both miRNA regulatory elements completely abolished the response to miR-494. These results together confirm that p190B is a direct target of miR-494 and 2 miRNA regulatory elements located between 56 and 90 bp downstream of the stop codon are the target sequences to which miR-494 binds.

3.6. Invasion-promoting action of miR-494 is mediated by p190B downregulation independently of RhoA activation

Having shown that miR-494 directly targets p190B and induces EGFR upregulation, we examined whether the suppression of p190B expression stabilizes EGFR like miR-494 does. Knocking down p190B expression by siRNA transfection delayed the turnover of EGFR after the stimulation with EGF (Fig. 6A), suggesting that miR-494-meidated p190B downregulation is responsible for the upregulation of EGFR by miR-494 through protein stabilization. Next, we tried to analyze the role of p190B downregulation in the promotion of invasion by miR-494. Downregulation of p190B expression by siRNA transfection resulted in increased MMP-2 secretion and invasion (Fig. 6B, C), which mimicked the effect of miR-494. To further confirm that miR-494 induces invasion mainly by targeting p190B, we tested whether the reconstitution of p190B expression by co-transfection with the Flag-p190B expression vector could suppress the invasion-promoting action of miR-494. Ectopic expression of Flag-p190B significantly inhibited both the

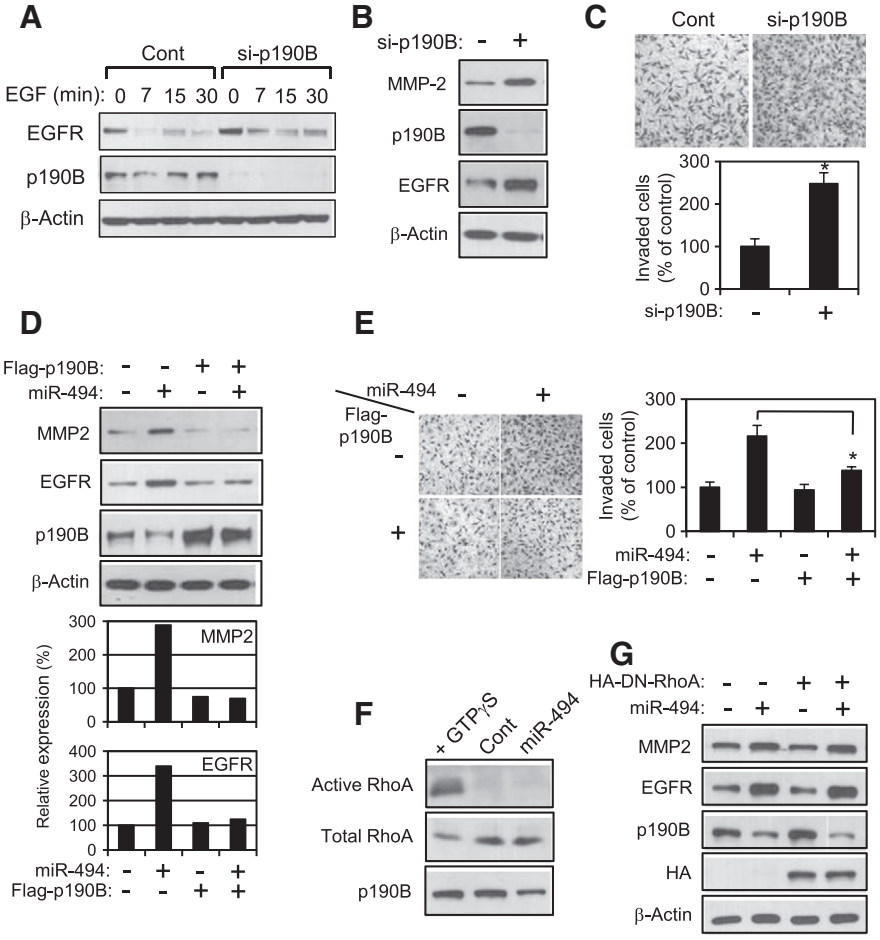

Fig. 6. Downregulation of p190B is essential for miR-494-promoted invasion independent of the regulation of RhoA activity. (A) Knocking-down p190B expression delays the turnover of EGFR. The cells transfected with control or si-p190B were pretreated with cycloheximide (CHX, 20 μg/mL) for 1 h, and then stimulated with EGF (100 ng/mL). The cells were harvested at the indicated times and analyzed for EGFR level by Western blotting. (B) Knocking-down p190B expression enhances MMP-2 secretion accompanied by the increase of EGFR expression. (C) Knocking-down p190B expression promotes the invasion of U-251 cells. *, $P < 0.001$ ($N = 3$). (D) Reconstitution of p190B expression suppresses MMP-2 secretion and EGFR upregulation induced by miR-494. U-251 cells were transfected with a combination of miR-494 and Flag-p190B expression vector. The conditioned media and cell lysates were prepared 48 h later and used to determine the MMP-2 and EGFR levels by using Western blot analysis. (E) Reconstitution of p190B expression suppresses the invasiveness of U-251 cells promoted by miR-494. U-251 cells transfected with a combination of miR-494 and Flag-p190B expression vectors were seeded onto Matrigel-coated filters to perform the invasion assay. *, $P < 0.001$ ($N = 3$). (F) Downregulation of p190B by miR-494 does not lead to RhoA activation. The cell lysates were prepared from U-251 cells transfected with control or miR-494, and subjected to RhoA pull down assays using recombinant Rhoetekin RBD agarose beads. The RhoA pulled down by the beads was analyzed by Western blotting. The cell lysate prepared from the GTPγS-treated cells was included as a positive control. (G) Dominant negative mutant of RhoA does not affect MMP-2 secretion and EGFR upregulation induced by miR-494. U-251 cells were transfected with a combination of miR-494 and the expression vector for dominant negative (T19N) RhoA, and the conditioned media and cell lysates were prepared for Western blot analysis.

upregulation of EGFR and MMP-2 secretion induced by miR-494 expression (Fig. 6D). The invasion assay also showed that Flag-p190B expression significantly suppressed miR-494-induced invasion (Fig. 6E). Together, these results strongly suggest that p190B downregulation is a critical step in the invasion promoted by miR-494. Since p190B has an activity to enhance returning of active GTP-bound Rho GTPases to the basal inactive GDP-bound state, we tested whether the action of miR-494 through downregulation of p190B involves RhoA activation. Contrary to expectations, transfection with miR-494 did not result in the activation of RhoA (Fig. 6F). Accordingly, the upregulation of EGFR and MMP-2 secretion induced by miR-494 expression were not affected by overexpression of dominant negative mutant RhoA (Fig. 6G), thereby suggesting that the negative regulation of EGFR by p190B is achieved independently of the catalytic activity as a RhoGAP to inactivate RhoA.

4. Discussion

Growing evidence suggests that aberrant miRNA expression is closely linked to various types of cancer, and numerous miRNAs play important roles in various processes related to cancer biology, such as apoptosis and neoplastic transformation [16,24]. In this study, we identified miR-494 as an IR-inducible miRNA that promotes invasion by upregulation of EGFR expression and subsequent activation of downstream ERK and Akt signaling in glioma cell lines. We further demonstrated that p190B is the direct target of miR-494, downregulation of which is responsible for the EGFR upregulation by miR-494 through protein stabilization.

Many groups have reported a variety of miRNAs having invasion-suppressing or -promoting functions. Recent studies have demonstrated that many miRNAs such as miR-107, miR-383, miR-134, miR-15b, miR-152, and miR-146b-5p inhibit the invasion of various cancer cells by directly targeting the signaling molecules such as Notch2, IGFR1, Nanog, neuropilin2, MMP-3, and EGFR, respectively [25–29]. Comparatively, several invasion-promoting miRNAs have also been reported. Specifically, miR-92b and miR-24 promote the proliferation and invasion of glioma through the regulation of Wnt/β-catenin signaling via targeting Nemo-like kinase (NLK) and suppression of tumorigenicity 7-like (ST7L), respectively [30,31]. miR-10b induces glioma cell invasion by regulating MMP-14 and uPAR expression via the direct targeting of HOXD10 [32]. Unlike these miRNAs, miR-494 was demonstrated to

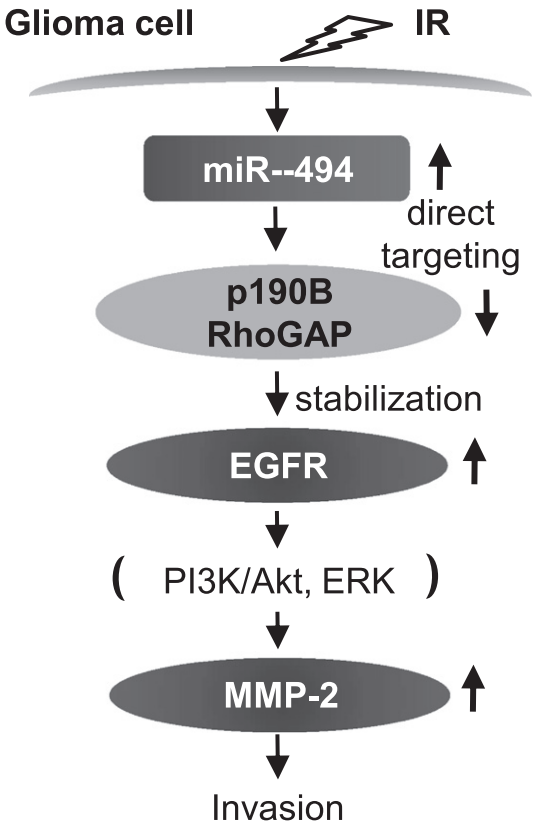

Fig. 7. Schematic summary for the invasion-promoting action of IR-inducible miR-494 through EGFR upregulation by directly targeting p190B in glioma cells.

induce glioma invasion through MMP-2 activation via upregulating EGFR expression in our study.

The upregulation of EGFR expression is often associated with carcinogenesis, and the resistance to tumor treatment, and can be achieved by multiple mechanisms. Amplification of the *EGFR* gene located on chromosome 7 is also a common genetic feature of gliomas and is known to be associated with shorter survival in patients who underwent a variety of treatments [33,34]. The U-251 cell line is also known to harbor *EGFR* gene amplification [35]. Our data indicates that miR-494 can lead to further upregulation of EGFR expression by protein stabilization even in a U-251 cell line that already expresses a high level of EGFR due to gene amplification. On the other hand, the miR-494 inhibitor reduced EGFR levels and invasion in U-251 cells, suggesting that targeting miR-494 can be a good strategy to control invasiveness of gliomas that commonly harbor *EGFR* amplification.

Besides gene amplification or transcriptional induction of *EGFR*, EGFR abundance at the cell surface can be regulated by various mechanisms at multiple stages. After EGFR activation by ligand binding, the Cbl protein mediates initial ubiquitination of EGFR, which targets it for endocytosis [36], and a variety of proteins including endosomal Rab proteins, Mig6, and Vav2 are known to regulate the trafficking from early endosomes to lysosomes and the subsequent degradation of EGFR [13,14,37,38]. Vav2, a GEF for Rho GTPases, positively regulates the stability of EGFR by delaying the internalization and degradation of EGFR [14]. Since p190B is a RhoGAP that plays an opposite role to Vav2 in the regulation of Rho activity, we analyzed whether p190B is a direct target of miR-494 and involved in the EGFR stabilization and invasion promotion by miR-494. Our results showed that p190B is the direct target of miR-494 and the downregulation of p190B is the main mechanism used by miR-494 to upregulate EGFR and promote glioma cell invasion. However, EGFR upregulation by miR-494 differed from that by the action of Vav2. In our results, miR-494 did not affect the kinetics of EGFR internalization and seemed to mainly suppress the lysosomal degradation of EGFR, which is in contrast to the case of Vav2 overexpression that delays both internalization and degradation of EGFR. EGFR stabilization through p190B downregulation by miR-494 was not accompanied by RhoA activation in our study using U-251 cells, while it was shown that RhoA is necessary for Vav2-mediated EGFR stabilization in HeLa cells. Several contradictory reports suggest that the function and mechanism of the major RhoA regulator p190 RhoGAP can vary depending on the cellular context and the processes involved. A study has demonstrated that p190 RhoGAP reduces pancreatic cancer cell invasion and metastasis by inactivating Rho GTPases [21]. In contrast, p190B was shown to have pro-tumorigenic functions during MMTV-Neu-induced mammary tumor formation and metastasis by enhancing cell adhesion and invasion through a Rac1/ROS dependent mechanism [39]. During matrix remodeling and angiogenesis, p190B mediates the expression of MMP-2 and MT1-MMP in endothelial cells independently of RhoA activation [40]. This is in contrast to our result that p190B downregulation enhances MMP-2 secretion in U-251 cells. It is possible that in U-251 cells, EGFR accumulation by miR-494-mediated p190B downregulation may dominate the otherwise opposite effects of p190B downregulation, consequently leading to the enhancement of MMP-2 activation and invasion. The mechanism of how p190B regulates the stability of EGFR independently of RhoA inactivation remains to be elucidated.

While this study was under way, several reports on the various functions of miR-494 have been published. miR-494 exerts cardioprotective effects against ischemia/reperfusion-induced injury, although both proapoptotic proteins (PTEN, ROCK1 and CaMKI) and antiapoptotic proteins (FGFR2 and LIF) are targeted by miR-494 [41]. miR-494 is regulated by ERK1/2 and induces TRAIL resistance in non-small-cell lung cancer through downregulation of BIM [42]. TGFβ1-induced miR-494 plays a key role in the accumulation and functions of myeloid-derived suppressor cells, which support tumor angiogenesis and metastasis, via targeting PTEN [43]. While these studies can be regarded to have demonstrated the cytoprotective or oncogenic characteristics of miR-494, there are also contrasting reports that demonstrate miR-494 is a tumor suppressor. miR-494 plays a suppressive role in IR-induced angiogenesis, which is associated with the MMP-9-miR-494-syndecan-1 regulatory loop in medulloblastoma cells [44]. In cholangiocarcinoma cells, miR-494 induces G2/M arrest by targeting many proteins involved in the G2/M transition such as CDC2, CDC20, and cyclin B1 [45]. miR-494 inhibits the cell proliferation of gastrointestinal stromal tumor by targeting KIT expression [46]. It is not uncommon that a miRNA plays opposite roles depending on the cellular context, because a single miRNA can simultaneously regulate a multitude of different specific target proteins. Likewise, the research implies that miR-494 has ambivalent functions. Glioma cells were not included in the list of cellular contexts in these previous studies analyzing the various functions of miR-494. In our study, the proliferation or cell cycle of U-251 glioma cells were not significantly affected by miR-494, but the effect of miR-494 on invasion through EGFR upregulation was significantly promotive in the U-251 cells. This suggests that aiding the promotion of invasion may be a main function of miR-494 in gliomas. Considering the previous report that glioma invasion is enhanced by IR through EGFR upregulation [7], the induction of miR-494 expression may be a key step in the promotion of glioma invasion by IR. Therefore, miR-494 is an attractive candidate target to control metastasis after radiotherapy against brain tumor.

In this study, we revealed a novel miR-494-mediated mechanism involved in the upregulation of EGFR expression and induction of invasion that entails the direct targeting of p190B and the subsequent stabilization of EGFR (Fig. 7). This is a new mechanism for regulating EGFR expression during the induction of invasion. Our findings will help develop a strategy to counteract the high invasiveness of glioma during cancer treatments like radiotherapy.

Acknowledgments

This work was supported by a grant from Nuclear Research & Development Program funded by the Korean Ministry of Science, ICT and Future Planning.

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